Inhibition of hepatitis C virus RNA translation by antisense bile acid conjugated phosphorothioate modified oligodeoxynucleotides (ODN)

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Abstract: BACKGROUND: The 5'-noncoding region (5'NCR) of the HCV-genome comprises an internal ribosome entry site essential for HCV-translation/replication. Phosphorothioate oligodeoxynucleotides (tS-ODN) complementary to this region can inhibit HCV-translation in vitro. In this study, bile acid conjugated tS-ODN were generated to increase cell-selective inhibition of 5'NCR-dependent HCV-translation. METHODS: Different bile acid conjugated tS-ODN complementary to the HCV5'NCR were selected for their inhibitory potential in an in vitro transcription/translation assay. To analyze OATP (organic anion transporting polypeptides)-selective uptake of bile acid conjugated ODN, different hepatoma cells were stably transfected with the OATP1B1-transporter and primary human hepatocytes were used. An adenovirus encoding the HCV5'NCR fused to the luciferase gene (Ad-GFP-NCRluc) was generated to quantify 5'NCR-dependent HCV gene expression in OATP-overexpressing hepatoma cells and in vivo. RESULTS: A 17mer phosphorothioate modified ODN (tS-ODN4_13)$complementary to HCV 5'NCR was able to inhibit 5'NCR-dependent HCV-translation in an in vitro transcription/translation test system by more than 90\% and it was also effective in cells containing the HCV subgenomic replicon. Conjugation to taurocholate (tS-ODN4_13T) significantly increased selectivity for expressing HepG2-cells compared to parental HepG2-cells. Correspondingly, tS-ODN4_13T significantly inhibited HCV derived OATP1B1-expressing HepG2- or CCL13-cells up to 70\% compared to unconjugated tS-ODN and compared to mismatch ODN. In vivo, tS-ODN4_13T showed also a trend to block 5'NCR-dependent HCV gene expression. CONCLUSIONS: The tested taurocholate conjugated 17mer antisense ODN complementary to HCV 5'NCV showed an increased and selective uptake in OATP-derived cells through OATP-mediated transport resulting in enhanced specific inhibition of HCV gene expression in vitro a

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Inhibition of hepatitis C virus RNA translation by antisense bile acid conjugated phosphorothioate modified oligodeoxynucleotides (ODN).

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Abbreviations:

Ad: adenovirus vector, FITC: fluorescein isothiocyanate, GFP: green fluorescent protein, HCV: hepatitis C virus, MOI: multiplicity of infection, 5'NCR: 5'-noncoding region of HCV, IRES: internal ribosomal entry site, OATP: organic anion transporting polypeptide, ODN: oligodeoxynucleotide(s), RNase: ribonuclease, RLU: relative light units, tS-ODN: phosphorothioate oligodeoxynucleotide, tS-ODN(T): taurocholate conjugated oligodeoxynucleotide

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Abstract

Background: The 5'-noncoding region (5'NCR) of the HCV genome comprises an internal ribosome entry site essential for HCV-translation/replication. Phosphorothioate oligodeoxynucleotides (tS-ODN) complementary to this region have been shown to inhibit HCV-translation *in vitro*. Thus, in this study, bile acid conjugated tS-ODN were generated to increase cell-selective and specific inhibition of 5'NCR-dependent HCV translation.

Methods: Different bile acid conjugated tS-ODN complementary to the HCV5'NCR were generated and selected for their inhibitory potential in an *in vitro* transcription/translation assay. An adenovirus encoding the HCV5'NCR fused to the luciferase gene (Ad-GFP-NCRluc) was generated to quantify 5'NCR-dependent HCV gene expression in cell culture and *in vivo*. The hepatoma cells HepG2 and CCL13 were stably transfected with the OATP1B1 transporter to analyze OATP-selective uptake of FITC-marked bile acid conjugated ODN by flow cytometry.

Results: In the *in vitro* transcription/translation assay, a 17mer taurocholate conjugated ODN (tS-ODN4_13T) showed the best inhibition of 5'NCR-dependent HCV translation. The inhibitory capacity of the selected 17mer antisense sequence was confirmed in Huh7 cells containing the HCV subgenomic replicon. In OATP1B1-expressing HepG2 or CCL13 cells, which were transduced with Ad-GFP-NCRluc, tS-ODN4_13T treatment significantly reduced 5'NCR-dependent

luciferase expression compared to the mismatch-ODN by 70% and 57%, respectively. *In vivo*, bile acid conjugated ODN also showed a trend to block 5'NCR-dependent HCV gene expression by 40%. Flow cytometry revealed an approx. two-fold increase of enhanced uptake of FITC-conjugated-tS-ODN4_13T in OATP1B1-HepG2 cells compared to parental HepG2 cells. Moreover, FITC-taurocholate conjugated tS-ODN were not taken up by non-hepatic cells, suggesting selective ODN entry into liver-derived cells.

Conclusions: Bile acid conjugation with taurocholate can increase selective ODN uptake by liver-derived cells through OATP transports, enhancing the specific inhibition of the HCV gene expression in different test systems. Thus, this approach may represent a new strategy for the selective control of HCV due to antisense ODN.

Summary 'box'

What is already known about this subject?

- Hepatitis C virus (HCV) is a major cause for chronic liver disease, cirrhosis and hepatocellular carcinoma, but current standard therapy remains unsatisfactory.
- The 5'-noncoding region (5'NCR) of the HCV genome comprises an internal ribosome entry site which is essential for HCV translation/replication.

- Antisense based strategies, such as phosphorothioate oligodeoxynucleotide (tS-ODN), complementary to the HCV5'NCR have been shown to inhibit HCV translation *in vitro*.
- However, major obstacles in the therapeutic application of antisense nucleotides are poor tissue delivery and cell membrane transport.

What are the new findings?

- A 17mer ODN sequence complementary to HCV5'NCR is able to inhibit 5'NCR-dependent HCV-translation in an *in vitro* transcription/translation test system by more than 90% and it is also effective in Huh7 cells containing the HCV subgenomic replicon.
- Conjugation of this 17mer phosphorothioate modified oligodeoxynucleotide to taurocholate significantly increases selective ODN uptake by OATP (organic anion transporting polypeptides) expressing liver-derived cells without transfection reagent.
- Taurocholate conjugated antisense tS-ODN with the 17mer sequence complementary to HCV5'NCR results in a specific and more effective inhibition of HCV gene expression in OATP-expressing liver-derived cells than unconjugated tS-ODN in cell culture and *in vivo*.

How might it impact on clinical practice in the foreseeable future?

- Taurocholate conjugated antisense ODN is a novel approach to improve delivery and efficacy of antisense nucleotides into the liver.

The tested taurocholate conjugated 17mer antisense ODN complementary to HCV5'NCV represents a promising strategy for the control of hepatitis C infection. It should be tested for its inhibitory effects in patients with chronic hepatitis C.

Introduction

Hepatitis C virus (HCV) is a major cause of liver disease, affecting over 200 million patients worldwide. Despite progress in HCV treatment, the overall response of a combined therapy with ribavirin and pegylated interferonα in patients with HCV genotype 1 or 4 remains unsatisfactory (1). Novel compounds against HCV-NS3 protease or HCV-NS5B RNA have been developed in recent years showing high antiviral activity (2). However, rapid HCV drug resistance to these agents considerably limits their efficacy. Thus, development of novel therapeutic strategies is called for.

HCV is a positive-strand RNA virus of approximately 9,600 nucleotides (nt). HCV isolates show a considerable sequence diversity leading to subdivision into six HCV genotypes (3, 4). At the 5'-end, the HCV genome contains a noncoding region (5'NCR) of 341nt, which is the most highly conserved region among all HCV strains (5, 6). It forms a stable secondary structure which contains an internal ribosome entry site (IRES) necessary for HCV translation/replication, representing an ideal target for antisense approaches.

In fact, we and others showed that phosphorothioate modified antisense oligodeoxynucleotides (ODN), RNA interference (siRNA), antisense RNA or hammerhead ribozymes complementary to this region can effectively inhibit HCV translation (7-11).

However, poor specific tissue delivery and the transport through cell membranes are major obstacles in the therapeutic application of antisense nucleotides (12). Various strategies using liposomes, nanoparticles, cholesterol conjugates or cell-penetrating peptides have been developed to enhance ODN or siRNA delivery. However, their tissue specificity and use *in vivo* remain significantly limited (13-16).

Two different transport systems are involved in the uptake of bile acids of hepatocytes: the Na^+-taurocholate-cotransporting polypeptide (NTCP) and several multispecific organic anion transporting polypeptides (OATPs) (17-19). Conjugation of several therapeutic drugs to bile acids has been shown to increase uptake into hepatoma cells via these transport systems (20, 21). Thus, conjugation of ODN to bile acids may also increase a liver-specific and more effective inhibition of HCV translation of antisense ODN.

In this study, conjugation of phosphorothioate-modified antisense oligonucleotides to taurocholate allowed selective ODN uptake in liver-derived cells and effective inhibition of HCV translation in different test systems, pointing to a promising new approach in the control of HCV infection.

Material and Methods

Chemical synthesis of bile acid conjugated oligonucleotides (ODN).

Starting from the previously described phosphorothioate modified 23mer ODN complementary to the nucleotides 326-348 of HCV5'NCR (tS-ODN4), which was identified as a potent inhibitor of the HCV translation (10), several shortened antisense ODN were generated for subsequent bile acid conjugation (Tab.1).

tS-ODN4	**5'**-T GC**T CAT GGT GCA CGG TCT A**CG A-**3'**	23mer
tS-ODN4_11	**5'**-T CAT **GGT GCA CGG TCT A**CG A-**3'**	20mer
tS-ODN4_13	**5'**-T GG**T GCA CGG TCT A**CG A-**3'**	17mer
tS-ODN4_26	**5'**-T CA**T GGT GCA** CGG **T-3'**	14mer
tS-ODN4_27	**5'**-T CA**T GGT** GCA **C-3'**	11mer
tS-ODN4_28	**5'**-T CA**T** GGT **G-3'**	08mer
tS-ODN4_30	**5'**-CT C**A**T GG**T-3'**	08mer
tS-ODN4_31	**5'**-GCT **C**AT G**G-3'**	08mer
tS-ODN4_33	**5'**-T GC**T** CAT **G-3'**	08mer
tS-ODN4_34	**5'**-AT G**G**T GC**A-3'**	08mer

Tab.1. Shortened ODN derived of tS-ODN4 (23mer) complementary to HCVRNA nucleotides 326-348. t: terminally-modified; S: phosphorothioate; nucleotides flanking the modified phosphate in 3' direction are in bold.

After testing their inhibitory capacity, tS-ODN4_13 was chosen for conjugation to cholate (C), taurocholate (T) or cholesterol (X) as

described previously (22), (Fig. 1A). Briefly, synthesis of cholic and taurocholic acid phosphoramidites started from commercially available cholic acid. In a sequence of seven reaction steps, 3α-(2-hydroxyethoxy)cholic acid could be obtained. The 2-hydroxyethoxy linker was introduced at the 3α-hydroxyl group, where it has least influence on substrate recognition by hepatic bile acid transporters (23). The analogous taurocholic acid phosphoramidite starting from 3α-(2-hydroxyethoxy)cholic acid was achieved via an amidation reaction with 2-aminoethane-ethyl-sulfonate. ODN synthesis was carried out on a PerSeptive Biosystems DNA synthesizer. To increase the nuclease stability of ODN, thioate modifications were introduced at three phosphodiesters at each end. In several control ODN, fluorescein isothiocyanat (FITC) was covalently attached to the ODN at the 3´-position. ODN were purified by RP-HPLC. FITC-labeled ODN were additionally purified by preparative polyacrylamide gel electrophoresis. All compounds were characterized by analytical RP-HPLC, analytical PAGE and electrospray ionization mass spectrometry(24).

Cell lines. 911 cells are human embryonic retinoblasts used to propagate E1-deleted adenoviruses (25). The human hepatoma cells HepG2 (ATCC HB8065) and CCL13 (ATCC CCL-13) were stably transfected with the OATP1B1 transporter to study selective delivery of bile acid conjugated ODN. The previously described Huh7 cell clone

Con1/Luc-ubi-neo/ET containing the HCV subgenomic replicon of genotype 1b was used for inhibition experiments (26). Kidney-derived cells HEK-293 (ATCC CRL-1573) and colorectal adenocarcinoma cells HT29 (ATCC HTB-38) served as control. Cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum (Life Technologies, Karlsruhe, Germany), 100 units/ml of penicillin and 100 µg/ml of streptomycin (Sigma, Taufkirchen, Germany). The stable transfected cells and the cell clones containing the HCV replicon were selected with neomycin (PAA, Cölbe, Germany).

Plasmids. Plasmids pCMVNCRluc and pT7NCRluc contain HCV5'NCR and the first 66 nt of the core gene fused in frame to the firefly luciferase sequence and have been previously described (10).
The adenoviral backbone vector pAdEasy-1 and the transfer vector pAdTrackCMV, which possesses a GFP-gene, kindly provided by Dr. B. Vogelstein, University of Baltimore, USA (27), were used to generate replication-deficient adenoviruses. Full-length OATP1B1-cDNA was isolated from a human liver Rapid-Screen library panel (OriGene Technologies) and cloned into the plasmid pCMV6-XL4 as described previously (28). OATP1B1-cDNA was cut out of pCMV6-XL4 and cloned into the expression vector pcDNA3.1/V5-His (Invitrogen, Karlsruhe, Germany) to generate pcDNA3.1/V5-His-OATP1B1.

In vitro **transcription/translation assay.** The *in vitro* transcription/translation test system has been described previously (10). Briefly, 1 µg of linearized pT7NCRluc was incubated with T7RNA-Polymerase (Promega, Mannheim, Germany) at 37°C for 1 h. 50ng of DNA-free RNA was used for translation into a commercially available rabbit reticulocyte lysate according to the manufacturer's instructions (Promega). ODN were added to the translation mixture and incubated at 75°C for 10 min and allowed to cool at room temperature for 1 h. Next, luciferase assay was performed.

Construction of adenoviral plasmids and production of virus stocks. The HCV-luciferase sequence NCRluc was cut from pCMVNCRluc and cloned into pAdTrackCMV. The generated plasmid pAdTrackCMV-NCRluc was recombined with pAdEasy-1 according to the published protocol by He *et al*. (27). Finally, recombinant plasmids were transfected into 911 cells to generate Ad-GFP-NCRluc viruses (Fig. 1B).

Generation of stably OATP1B1-transfected cells. Stable transfections were performed with lipofectamine according to the manufacturer's instructions. Briefly, HepG2 cells and CCL13 cells were grown up to a 90% confluence. 20 µg pcDNA3.1/V5-His-OATP1B1 was mixed with 20 µl lipofectamine and FCS-free medium (Optimem,

Invitrogen) and incubated for 45 min. Lipofectamine/DNA mixture was added to the cells for 4 h. 72 h later cells were cultured under G418 selection (800 µg/ml) for four weeks.

Polymerase chain reaction (PCR). To demonstrate 5'NCR transcription in HepG2-OATP1B1 and CCL13-OATP1B1 cells after transduction with Ad-GFP-NCRluc, total cellular RNA was isolated with the RNA Isolation Kit (Sigma, Taufkirchen, Germany) according to the manufacturer's instructions. PCR was performed using the following primers: forward, HCV5´NCR 5'-GCTCTAGACCCAAGCTTGCCAGCC-3' and reverse, HCV5´NCR 5'-GGCCGGTACCGACGTCCTGTGGG-3'. 1 µg of DNA-free RNA was mixed with the primers and denatured at 75°C for 10 min. Subsequently, reverse transcription was performed at 37°C for 1 h. Generated cDNA served as template for the amplification reaction.

To confirm OATP1B1 transcription, total cellular RNA was isolated from stable transfected HepG2-OATP1B1 and CCL13-OATP1B1 cultures. The following primers, corresponding to bp 1188-1211 and 1664-1688 of the OATP1B1 cDNA, were used for reverse transcription: forward, 5'-AGAGCAACAGTATGGTCAGCCTTC-3' and reverse, 5'-AAGCATCATCTCTTGGGCATTCAC-3'. 50 ng of RNA were reverse transcribed, followed by PCR amplification as described above.

Protein extraction and luciferase assay. Cells were solubilized in lysis buffer (25 mM Tris-H_3PO_4, 2 mM CDTA, 10%DTT and 1%Triton X-100). Lysates were cleared by centrifugation, and protein concentration was determined with the Bradford protein assay (BioRad, Munich, Germany) according to the manufacturer's instructions. Luciferase activity was determined with the Luciferase Reporter System (Promega, Mannheim, Germany) according to the manufacturer's instructions. The relative light units (RLU) were measured during 30 sec in a luminometer (Berthold, Pforzheim, Germany).

Western blot analysis of OATP1B1 expression in hepatoma cells. Expression of OATP1B1 was determined by Western blot using standard protocols. The primary monoclonal antibody mESL kindly provided by Prof. Dietrich Keppler, University of Heidelberg (Germany) (29) and a horse peroxidase (HRP)-conjugated secondary goat anti-rabbit antibody (Sigma, Munich, Germany) were used. Visualization was achieved by enhanced chemoluminescence (SuperSignal Chemiluminescent-Substrate, Perbio-Science, Bonn, Germany).

Flow cytometry analysis. Efficiency of cell transduction and GFP expression in hepatoma cells after transduction with Ad-GFP-NCRluc were measured by flow cytometry (Coulter Epics XL-Cytometer,

Immunotech, Krefeld, Germany). FITC-marked bile acid conjugated ODN served to analyze the cellular ODN uptake by flow cytometry.

Adenoviral transduction of hepatoma cells and inhibition experiments. HepG2-OATP1B1, CCL13-OATP1B1 or Huh7 cells containing a HCV subgenomic replicon genotype 1b were seeded onto 24-well plates and grown for 48 h. For experiments in HepG2-OATP1B1 and CCL13-OATP1B1 cells, cells were transduced with Ad-GFP-NCRluc with MOI 50 in DMEM supplemented with 2% horse serum for 2 h. Six hours after transduction, 2-6 µM ODN was added to the culture medium without any transfection reagent. For experiments with unconjugated ODN, ODN were mixed with 2 µl lipofectamine and 200 µl Optimen and added to the cells for 24 h. For inhibition experiments in the Huh7 cells with the HCV replicon, 4 µM ODN was added to the culture medium.

Imaging and quantification of bioluminescence. Animals were injected intravenously with the reporter vector Ad-GFP-NCRluc ($5x10^7$pfu/mouse). Luciferase was measured *in vivo* by live imaging using a highly sensitive, cooled charge-couple device camera (IVIS 200 Xenogen, Alameda, CA, USA). For imaging, luciferin (Synchem, Alternburg, Germany) was injected intraperitonally at a dose of 150 mg/kg body weight. After 5 min, light emitted from the mice was imaged

and electronically displayed using a Living Image Software (Xenogen, Ivis).

Statistical analysis. For descriptive statistics, means and standard errors were noted. Paired t-test was used for statistical significance. A p-value of less than 0.05 was considered significant.

Results

Inhibition of HCV translation by bile acid conjugated phosphorothioate ODN *in vitro*.

Inhibitory activity of shortened phosphorothioate tS-ODN.

The previously identified 23-base phosphorothioate modified oligonucleotide tS-ODN4 as potent inhibitor of HCV translation (10) was chosen for bile acid modification. To increase cell uptake of ODN after bile acid conjugation, we shortened the ODN sequence and generated several ODN derived from tS-ODN4 (s. Tab. 1). The effect of ODN length on their inhibitory potential was compared to the potent tS-ODN4 in an *in vitro* transcription/translation assay. As shown in Fig. 2A, tS-ODN shortened to 20-17bp could still inhibit translation of the fusion HCV-RNA by >95%. With regard to the number of nucleotides, 17bp ODN (tS-ODN4_13) was the shortest sequence with the best inhibitory effect on the 5'NCR-dependent luciferase gene expression. This effect was HCV5'NCR-sequence specific, as the 17bp mismatch tS-ODN4_13(K) could not inhibit HCV-translation (Fig. 2D). Therefore, for further bile acid conjugation, the 17mer tS-ODN was chosen as described in the methods.

Further shortening of tS-ODN to 16-11bp resulted in lower inhibitory activity (<75%) and further shortening of ODN to 8bp in almost no inhibitory activity (<30%) (Fig 2B/C).

Effect of bile acid conjugation on the inhibitory activity of tS-ODN4_13.

As shown in Fig. 3A, taurocholate (T), cholate (C) and cholesterol (X) conjugated antisense tS-ODN4_13 showed strong inhibition rates of 5'NCR-dependent luciferase expression comparable to the inhibitions of unconjugated tS-ODN4_13 *in vitro*. Maximum suppression of HCV translation was achieved with the taurocholate conjugated ODN tS-ODN4_13T. In this case, 4 µM tS-ODN4_13T reduced the luciferase activity by >90% ($p<0.0001$). Taurocholate tS-ODN4_13 was also the most effective bile conjugated ODN at the lowest concentration tested (0.5 µM), leading to an inhibition of almost 80% ($p<0.0001$). Contrary, cholate and cholesterol-coupled ODN (tS-ODN4_13C and tS-ODN4_13X) reached maximum inhibition rates of 70% at 5 µM and no inhibitory effects at 0.5 µM.

Inhibition of HCV gene expression by bile acid conjugated tS-ODN4_13 in Huh7 cells containing a HCV subgenomic replicon.

The inhibitory effects of taurocholate conjugated tS-ODN4_13 could not be reproduced properly in Huh7 cells containing a HCV subgenomic replicon of genotype 1b (Con1/Luc-ubi-neo/ET) since Huh7 cells have a down-regulation of bile acid transports. As expected, uptake of FITC-marked tS-ODN4_13T was very low and correspondingly, almost no inhibitory effects were observed on HCV gene expression in this system (not shown). However, the cholesterol-coupled tS-ODN4_13X, which were highly incorporated by the Huh7 cells of the replicon

system (not shown) showed a significant inhibition of HCV translation of about 40% 48 h after ODN treatment compared to mismatch tS-ODN4_13XK ($p<0.0001$) (Fig. 3B), demonstrating the inhibitory capacity of the chosen 17mer ODN sequence in the replicon system.

Generation of OATP1B1 overexpressing hepatoma cells.

In order to test the inhibitory potential of taurocholate coupled tS-ODN4_13T in cell culture, the hepatoma cell lines HepG2 and CCL13 were used. In these cells, a decreased expression of bile acid transporters, such as OATP, has been described compared to hepatocytes (30). Therefore, HepG2 and CCL13 cells were stably transfected to overexpress the OATP1B1 transporter. By RT-PCR, the transcription of OATP1B1 in HepG2 and CCL13 was verified as a strong amplification of a 490bp product compared to a very low or no expression in wild type HepG2 or CCL13 cells (Figs. 4A/B). By Western blot analysis, two bands of 84 kDa and 60 kDa in both OATP1B1 overexpressing cell lines were detected (29), (Fig. 4C/D). The lower band corresponded to the core-glycosylated form of OATP1B1 (31). As expected, OATP1B1 was clearly reduced or not detectable in wild type HepG2 and CCL13 cells and no specific signal was detected in 911 cells (negative control).

Construction and characterization of a NCRluc-encoding adenovirus vector.

To quantify the inhibition of HCV translation by taurocholate conjugated antisense ODN in OATP-overexpressing HepG2 and CCL13 cells, we generated an adenovirus encoding HCV5'NCR fused to the luciferase gene as reporter vector (Ad-GFP-NCRluc) as described in methods. 48 h after transduction of HepG2-OATP1B1 and CCL13-OATP1B1 cells with Ad-GFP-NCRluc, target-RNA (407bp) was confirmed by RT-PCR (Fig. 5A). The 5'NCR-dependent luciferase activity was dose-dependent, reaching a peak 24 h after adenoviral transduction for all tested MOI (Figs. 5B/C).

Inhibition of 5'NCR-dependent HCV gene expression by taurocholate conjugated phosphorothioate ODN in cell culture.

Stably transfected HepG2-OATP1B1 and CCL13-OATP1B1 cells were transduced with Ad-GFP-NCRluc (MOI 50). Six hours later, 2-6 µM tS-ODN4_13T was added to the cell supernatant without any transfection reagent. GFP expression served to normalize transgene luciferase expression. As shown in Figs. 6A/B, tS-ODN4_13T reduced luciferase activity in a dose-dependent manner reaching inhibition rates of 31.9+/-0.9% with 2 µM ODN and 61.7+/-2.3% with 4 µM ODN in HepG2-OATP1B1 compared to the mismatch control (p=0.032 and p=0.012, respectively). Addition of 6 µM ODN induced strong inhibition of

5'NCR-dependent translation of 70.6+/-4.9% compared to mismatch ODN (tS-ODN4_13TK), (p=0.0037). Similarly, in CCL13-OATP1B1 cells, the maximum detected inhibition reached 57.4+/-0.45% (p=0.0042) 24 h after treatment with 6 µM tS-ODN4_13T.

Inhibition of 5'NCR-dependent HCV gene expression by unconjugated tS-ODN4_13 in cell culture.

In contrast, no significant inhibition of 5'NCR-dependent luciferase activity was detected after addition of unconjugated tS-ODN4_13 to the supernatant of OATP1B1-overexpressing HepG2 and CCL13 cells (not shown), indicating the OATP-selective cell delivery of taurocholate conjugated ODN in the previous experiment. When only a transfection reagent, such as lipofectamine, was applied, a moderate specific inhibition of HCV translation of approximately 35% with 4 µM tS-ODN4_13 was observed in CCL13-OATP1B1 cells (p=0.018), (Fig. 7B). In HepG2-OATP1B1 cells, despite the use of lipofectamine, no decrease of luciferase activity was detected (Figs. 7A).

Intracellular uptake of taurocholate-conjugated ODN.

To analyze intracellular uptake of taurocholate coupled ODN, FITC-labeled tS-ODN4_13T were generated and used for flow cytometry. As shown in Figs. 8A/B, an about twice as high uptake of taurocholate conjugated tS-ODN4_13T was measured in OATP1B1-overexpressing

HepG2 cells (>90% of cells) compared to wild type HepG2 cells (only 44% of cells). Moreover, intensity of fluorescence was approximately 1log higher in HepG2-OATP1B1 cells than in HepG2 cells, also indicating an enhanced concentration of taurocholate conjugated ODN/cells. Surprisingly, FITC-non-conjugated ODN were also taken up by a relatively high percentage of HepG2-OATP1B1 cells (about 75%). Yet, intensity of fluorescence was much lower compared to taurocholate conjugated ODN, explaining the failure of inhibition of unconjugated ODN in HepG2 cells.

As expected, taurocholate conjugated tS-ODN were not taken up by a number of tested non-hepatic human cell lines, such as A549 cells (lung carcinoma) or 911 cells (retinoblastoma), suggesting a selective delivery of taurocholate coupled tS-ODN into liver-derived cells (Fig. 8C/D).

It has been shown that certain non-hepatic tissues also possess transporters for bile acids, notably the kidney and the bowel. Correspondingly, an uptake of FITC-labeled taurocholate tS-ODN was detected in approximately 30% of HEK-293 cells (kidney-derived cells), and in about 35% of HT29-cells (colorectal adenocarcinoma cells) (Fig. 8E/F).

Inhibition of HCV gene expression by antisense bile acid conjugated ODN *in vivo*.

For *in vivo* evaluation of bile acid conjugated oligonucleotides tS-ODN4_13T, we used the Ad encoding 5'NCR-luciferase fusion RNA (Ad-GFP-NCRluc). After intravenous (i.v.) application of Ad-GFP-NCRluc ($5x10^7$ pfu/mouse), a high HCV luciferase expression could be tracked by whole body imaging bioluminescence in the liver of the mice. For inhibition experiments, mice were i.v.-treated with 20 mg ODN/kg body weight. As shown in Fig. 9, taurocholate coupled antisense tSODN showed a trend to higher inhibition of NCR-dependent luciferase of about 40%, compared to mismatch ODN and unconjugated ODN.

Discussion

In this study, we show that taurocholate conjugated antisense oligonucleotides (ODN) complementary to the 5'NCR of the HCV genome allow selective ODN uptake into liver-derived cells and high specific inhibition of HCV translation in different test-systems indicating a novel strategy in the control of HCV infection.

Design and testing of bile acid-coupled HCV-specific antisense ODN in vitro.

The highly conserved 5'NCR of the HCV genome necessary for the HCV translation/replication has been shown to be an ideal target sequence for different antisense approaches, such as antisense RNA, RNA interference (RNAi), ribozymes or ODN (7-11). Phosphorothioate modified ODN are the first generation of antisense drugs entering clinical trials for the treatment of patients with chronic HCV infection which show acceptable properties for drug development (32). In the past, we reported that a 23-base phosphorothioate ODN (tS-ODN4) complementary to the nucleotides 326-348 of HCV5'NCR was highly efficient in reducing viral translation *in vitro* (10).

However, a major limitation of the antisense technology - whether ODN, ribozymes or siRNA - is the specific tissue delivery. Phosphorothioate ODN are poorly incorporated into cells and usually a vehicle for tissue delivery is required. Several strategies, such as

liposomes, poly(ethylenglycol) (PEG)-conjugates, nanoparticles, electroporation, as well as cholesterol conjugates or cell-penetrating peptides, have been explored for tissue delivery of antisense molecules. However, these compounds either carry considerable cytotoxicity or their tissue specificity remains limited (13-16).

Conjugation of drugs as well as small peptide molecules and ODN by bile acid carriers has been described to augment uptake and selectivity of drug action, specifically in the liver (33, 34). Conjugation of a drug to a bile acid has also the theoretical advantage of improving delivery from the gut to the liver due to enterohepatic circulation. Moreover, bile acids appear to stabilize ODN against possible degradation (35). Thus, in this work, different bile acid conjugated phosphorothioate modified antisense ODN were generated and tested in order to achieve selective ODN uptake and more specific inhibition of HCV gene expression.

Reduction of the number of nucleotides can favor uptake of ODN in hepatocytes after conjugation with bile acids, while at the same time, shortened ODN can lose their inhibitory efficacy. Therefore, in the first part of this work, we analyzed several shortened ODN derived from the 20mer tS-ODN4 for their remaining antisense effects using an *in vitro* transcription/translation assay. Despite nucleotide reduction, a 17mer phosphorothioate modified ODN (tS-ODN4_13) complementary to nt 342-326 of the HCV genome achieved almost a complete block of luciferase expression (95%) comparable to the parental tS-ODN4. This

effect was HCV sequence-specific, since the mismatch ODN (tS-ODN4_13K) did not inhibit HCV translation. Further shortening of the 20mer tS-ODN4 considerably reduced the inhibitory capacity of ODN, suggesting that inhibition of gene expression was dependent on the number of nucleotides which complementarily bind to the target sequence on the 5'NCR of HCV. The specific hybridisation of single-stranded ODN to the complementary messenger RNA sequence by Watson-Crick base-pairing rules is a key mechanism of ODN blocking of the protein translation (36). Therefore, longer antisense sequences of nucleotides display increased nucleotide binding capacity and are more specific to its target, which explains the lower inhibitory effects of shortened ODN from tS-ODN4.

The 17mer ODN, tS-ODN4_13, was the least effective inhibitory sequence and was therefore chosen for bile conjugation with cholate, taurocholate and cholesterol. Interestingly, while cholate and cholesterol conjugation reduced the inhibitory capacity of tS-ODN4_13, taurocholate conjugation exhibited no negative effects on the inhibitory capacity of the 17mer ODN reaching inhibition rates of >90% of HCV-translation *in vitro.*

Inhibition of bile acid-coupled HCV-specific antisense ODN in the HCV replicon system.

In the past, a system based on subgenomic HCV replicons capable to replicate in the hepatoma cell line Huh7 was developed (26). However, hepatoma cells are known to have a down-regulation of bile acid transports (29, 30). Correspondingly, FITC-marked taurocholate conjugated tS-ODN4_13 were not taken up by Huh7 cells, explaining the failed inhibition of HCV translation of tS-ODN4_13 in the replicon system. However, high uptake of cholesterol-coupled FITC-marked ODN by Huh7 cell with HCV replicons was noted, suggesting a non-selective entrance of cholesterol ODN. Conjugation with cholesterol or other lipid moieties has been shown to enhance hepatic cell ODN uptake via lipoprotein-mediated endocytosis, explaining our results in the replicon system (37, 38). Correspondingly, cholesterol-coupled tS-ODN4_13X could moderately inhibit HCV gene expression in the HCV replicon system, indicating that the 17mer antisense sequence complementary to HCV5'NCR effectively inhibited HCV translation in the replicon system.

Cell culture and in vivo HCV test systems.

However, cholesterol-conjugated ODN showed less inhibitory capacity in the *in vitro* transcription/translation assay than taurocholate coupled ODN. Most importantly, as the goal of this work was to demonstrate a selective cell uptake of ODN due to bile acid transports experiments in cell lines with an adequate expression of bile acid transporters were

necessary to evaluate the efficiency of the potent taurocholate coupled ODN, tS-ODN4_13T.

The organic anion transporting polypeptide (OATP) is a family of transport proteins responsible for transmembrane transport of diverse endogenous and exogenous compounds. In contrast to other OATP members localized in a variety of tissues (liver, kidney, brain and intestine) (30, 39), OATP1B1 has been found selectively in hepatocytes. It is the most important OATP for liver-specific bile salt uptake (17, 40). For this reason, OATP1B1 was chosen to generate stably transfected hepatoma cells. After stable transfection of HepG2 and CCL13 with the OATP1B1-containing plasmid, an overexpression of OATP1B1 compared to the parental cell lines was observed.

For completion of our cell culture test system, we generated an adenoviral vector (Ad-GFP-NCRluc) able to express the HCV-5'NCR sequence fused to the luciferase gene. For this fusion construct, we previously demonstrated that expression of luciferase is related to IRES activity of HCV5'NCR (10), providing a sensitive tool for quantification of the antisense effects related to HCV5'NCR. An adenoviral system offers a high transgene expression in hepatoma cells, allowing rapid screening and quantification of the 5'NCR-dependent HCV gene expression in the generated OATP1B1-overexpressing hepatoma cells. Moreover, an adenoviral system offers the possibility to evaluate inhibitions of the HCV5'NCR-related

luciferase expression in the liver of animal models after application of adenoviral vectors via the tail vein (8).

Analysis of selective uptake of taurocholate conjugated tS-ODN in cell culture test system.

To analyze the selective OATP1B1-dependent uptake of taurocholate conjugated ODN we generated FITC-labeled ODN. In contrast to earlier results of Petzinger et al. (35), we show that bile acid conjugation can deliver ODN to liver-derived cells via bile acid transport. In fact, uptake of taurocholate conjugated tS-ODN in OATP1B1-overexpressing HepG2 cells was found to be about twice as high than in wild type HepG2 cells with low or non-existent expression of bile acid transporters. Moreover, taurocholate conjugated tS-ODN were not taken up by non-hepatic cell lines, indicating selective entry of bile acid conjugated ODN only into liver-derived cells with enough bile acid transporter expression. The moderate uptake of taurocholate conjugated ODN found in wild type HepG2 cells, but also in HEK-293 and HT29 cells, suggested contribution of other bile acid uptake transporters. Molecular evidence for the expression of both NTCP and different members of the OATP family in human hepatocellular carcinoma cells was found (30). Furthermore, OATP transporters are expressed in the gastrointestinal tract and in the kidney, explaining our observations in HEK293 and HT29 cells (41).

Analysis of inhibition of HCV translation by taurocholate conjugated antisense ODN in cell culture test system and in vivo.

Confirming our hypothesis, the increased uptake of taurocholate coupled ODN in OATP1B1-overexpressing hepatoma cells was translated into a higher and more efficient inhibition of the 5'NCRHCV-dependent translation, reaching inhibition rates of >70%. The antisense inhibition was HCV sequence-specific since the mismatch taurocholate coupled ODN did not inhibit HCV5'NCR-dependent luciferase expression. By contrast, no significant decrease of luciferase activity was observed with unconjugated antisense ODN when used without transfection reagent in HepG2-OATP1B1 cells and CCL13-OATP1B1 cells. Taurocholate conjugation also provided a more efficient antisense effect on inhibiting HCV translation than use of traditional strategies, such as liposome-transfected ODN. In fact, even when using lipofectamine unconjugated ODN showed only moderate specific inhibition of HCV translation in CCL13 cells and no inhibition in HepG2 cells.

These findings are of importance because taurocholate conjugation of antisense ODN may overcome the problems derived from use of viral methods or invasive systems for delivering antisense molecules into the liver. As it can also be applied to other liver-related target genes it represents a novel approach for antisense based strategies.

In vivo, mice treated with taurocholate coupled antisense ODN also exhibited a trend towards less expression of the 5'NCR-dependent luciferase activity in the liver than mice treated with unmodified ODN, confirming the data in cell culture. However, standard deviations were high between the animals. This was probably due to a host immune response to viral proteins expressed by adenoviral vectors *in vivo.* Nevertheless, this experiment provided an important proof-of-concept, namely that taurocholate conjugated antisense ODN can also inhibit HCV5'NCR-dependent luciferase gene expression without apparent toxicities *in vivo*.

In conclusion, we demonstrated that conjugation of ODN to bile acids such as taurocholate increases selective uptake of antisense ODN in bile acid transport expressing liver-derived cells. Taurocholate conjugated antisense ODN with sequences complementary to HCV5'NCR resulted in specific and effective inhibition of HCV gene expression. Thus, this approach may improve the use of antisense ODN into the liver and possibly represents a novel promising strategy for the control of hepatitis C virus infection.

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Figure legends

Figure 1

A. 5´-bile acid and 3´-fluorescein modified antisense ODN (17mer) complementary to HCV5'NCR.

B. Map of Ad-GFP-NCRluc with deletion of E1-, E3-genes, encoding two transgenes: the green fluorescent protein (GFP), and HCV5'NCR and 66 nt of core-sequence, fused to the firefly luciferase gene. LITR: left inverted terminal repeat, ES: encapsidation signal, CMV: cytomegalovirus immediate early promotor, MCS: multiple cloning site, Poly A: polyadenylation signal, Ad5ΔE1/ΔE3: human adenovirus type 5 sequence with deletion of E1- and E3-genes, RITR: right inverted terminal repeat.

Figure 2 Inhibitory activity of different shortened phosphorothioate modified ODN on HCV translation in an *in vitro* transcription/translation assay. All experiments were performed in triplicate. The percentage mean values and standard deviations are given.

A. Inhibition rate of shortened antisense tS-ODN to 20mer - - ● - -, 17mer —■— , 14mer – ■ - and 11mer—◆— compared to 23mer tS-ODN4 – ▲ –

B. Inhibition rate of shortened antisense tS-ODN to 18mer ,17mer , 16mer , to 15mer and 14mer compared to 23mer tS-ODN4, as control mismatch tS-ODN4K

C. Inhibition rate of several shortened antisense tS-ODN to 8mer: tS-ODN4_28 , tS-ODN4_30 , tS-ODN4_31 ,tS-ODN4_33 and tS-ODN4_34 compared to 23mer tS-ODN4, mismatch ODN tS-ODN4K (as control)

D. Inhibition rate of 17mer shortened ODN tS-ODN4_13 compared to 23mer tS-ODN4, as controls, mismatched ODN tS-ODN4K and tS-ODN4_13K were used.

Figure 3

A. Inhibitory activity of bile acid conjugated phosphorothioate modified antisense ODN on HCV-translation in an *in vitro* transcription/translation assay. Taurocholate , cholic acid and cholesterol conjugated ODN compared to unconjugated tS-ODN4_13 . Mismatch ODN tS-ODN4_13K (as control)

B. Inhibitory activity of cholesterol phosphorothioate modified antisense tS-ODN4_13X (17mer) on HCV translation in Huh7 cell clone Con1/Luc-ubi-neo/ET containing the HCV subgenomic replicons of genotype 1b. Luciferase was measured 24 h and 48 h after ODN

treatment. Mean and SE are shown. Black bars: tS-ODN4_13 (antisense); black bars: tS-ODN4_13K (mismatch).

Figure 4

A. Transcription of OATP1B1 confirmed by RT-PCR in HepG2-OATP1B1 cells. Line 1: marker, line 2: OATP1B1 in 911 cells (control) line 3: OATP1B1 in wild type HepG2 cells, line 4: OATP1B1 in HepG2OATP1B1 cells (clone I), line 5: OATP1B1 in HepG2OATP1B1 cells (clone II), line 6: OATP1B1 in HepG2OATP1B1 cells (clone III), line 7: beta actin in 911 cells, line 8: beta actin in wild type HepG2 cells, line 9: beta actin in HepG2OATP1B1 cells (clone I), line 10: beta actin in HepG2OATP1B1 cells (clone II), line 11: beta actin in HepG2OATP1B1 cells (clone III), line 12: OATP1B1 using plasmid pcDNA3.1/V5-HisOATP-C (positive control).

B. Transcription of OATP1B1 confirmed by RT-PCR in CCL13-OATP1B1 cells. Line 1: marker, line 2: OATP1B1 in 911 cells, line 3: OATP1B1 in CCL13OATP1B1 cells (clone A), line 4: OATP1B1 in CCL13OATP1B1 cells (clone B), line 5: OATP1B1 in wild type CCL13 cells, line 6: beta actin in 911 cells, line 7: beta actin in CCL13OATP1B1 cells (clone A), line 8: beta actin in CCL13OATP1B1 cells (clone B), line 9: beta actin in wild type CCL13 cells, line 10:

OATP1B1 using plasmid pcDNA3.1/V5-HisOATP-C (positive control). Only clone A was positive for OATP1B1.

C. Expression of OATP1B1 confirmed by Western blot in stably transfected HepG2-OATP1B1 cells. Lines 1 and 2: OATP1B1 in wild type HepG2 cells. Line 3: OATP1B1 in HepG2OATP1B1 (clone I). Line 4: OATP1B1 in HepG2OATP1B1 cells (clone II). Line 5 and 6: OATP1B1 in 911 cells (negative control).

D. Expression of OATP1B1 confirmed by Western blot in stably transfected CCL13-OATP1B1 cells, lines 1 and 2: OATP1B1 in CCL13OATP1B1 (clone A); lines 3 and 4: OATP1B1 in CCL13 cells (wild type); lines 5 and 6: OATP1B1 in 911 cells (negative control).

Figure 5

A. Confirmation of transcription of target RNA (HCV5'NCR) in stable transfected hepatoma cells after adenoviral transduction with Ad-GFP-NCRluc (MOI 50) by RT-PCR. Lines 1 and 7: marker; line 2: CCL13OATP1B1 cells; line 3: HepG2OATP1B1 cells, line 4: HepG2OATP1B1 using a second primer, line 5: plasmid pAdTrackCMVNCRluc, line 6: plasmid pCMVNCRluc (positive control).

B. Kinetic of the luciferase expression in HepG2-OATP1B1 cells after adenoviral transduction with Ad-GFP-NCRluc at different MOIs.

C. Kinetic of luciferase expression in CCL13-OATP1B1 cells after adenoviral transduction with Ad-GFP-NCRluc at different MOIs.

In both cases, luciferase activity was determined 8, 16, 24, 48 and 72 h after transduction with Ad-GFP-NCRluc: MOI 5 - - ● - - , MOI 25 – ■ –, MOI 50 —■—, MOI 100 —◆—, MOI 200 – ▲ –, (n=3).

Figure 6

A. Inhibition of HCV5'NCR-dependent luciferase gene expression in HepG2-OATP1B1 cells.

B. Inhibition of HCV5'NCR-dependent luciferase gene expression in CCL13-OATP1B1 cells.

To test the inhibitory potential of bile acid conjugated antisense tS-ODN4_13T, $4x10^4$ cells/well were seeded on a 24-well-plate. After 24 h, cells were transduced with Ad-GFP-NCRluc at MOI 50. Six hours later, tS-ODN4_13T were added in different concentrations (2, 4 or 6 μM) to the culture medium without any transfection reagent. Luciferase activity was determined 16, 24, 48 and 72 h later. As control, a mismatch antisense taurocholate conjugated ODN, tS-ODN4_13TK, was used. White bars: control ODN; black bars: antisense ODN. Mean and SE are shown (n=3).

Figure 7

A. Inhibition of HCV5'NCR-dependent luciferase gene expression in HepG2-OATP1B1 cells using unconjugated antisense tS-ODN4_13. **B.** Inhibition of HCV5'NCR-dependent luciferase gene expression in CCL13-OATP1B1 cells using unconjugated antisense tS-ODN4_13. In both experiments, unconjugated tS-ODN4_13 were transfected using lipofectamine. Luciferase activity was determined 16, 24, 48 and 72 h later. White bars: mismatch tS-ODN4_13; black bars: antisense tS-ODN4_13. Mean and standard error are shown (n=3).

Figure 8

Selective uptake of FITC-labeled taurocholate conjugated tS-ODN in different cell lines was analyzed by flow cytometry. 2 μM of FITC-labeled ODN was added to different cell types before flow cytometry was performed. **A.** Uptake of FITC-labeled ODN by HepG2 cells (wild type), **B.** by HepG2OATP1B cells, **C.** by A549 cells; **D.** by 911 cells; **E.** by HEK293 cells; **F.** by HT29 cells.

Figure 9

Inhibition of HCV5'NCR-dependent luciferase gene expression *in vivo*. $5x10^7$ pfu/mouse Ad-NCRluc was injected i.v.. Two hours later, 20 mg/kg body weight ODN was injected i.v. Luciferase activity was determined 8, 24, 48 and 72 h later.

A. Inhibition experiments using unconjugated ODN: White bars: mismatch tS-ODN4_13K; black bars: antisense tS-ODN4_13

B. Representative image of light emitted from two mice 24 h after i.v. injection of tS-ODN4_13K (left) or tS-ODN4_13 (right).

C. Inhibition experiments using taurocholate conjugated ODN: White bars: mismatch tS-ODN4_13TK; black bars: antisense tS-ODN4_13T

D. Representative image of light emitted from two mice 24 h after i.v. injection of tS-ODN4_13TK (left) or tS-ODN4_13T (right).

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Gonzalez-Carmona *et al*., figure 1

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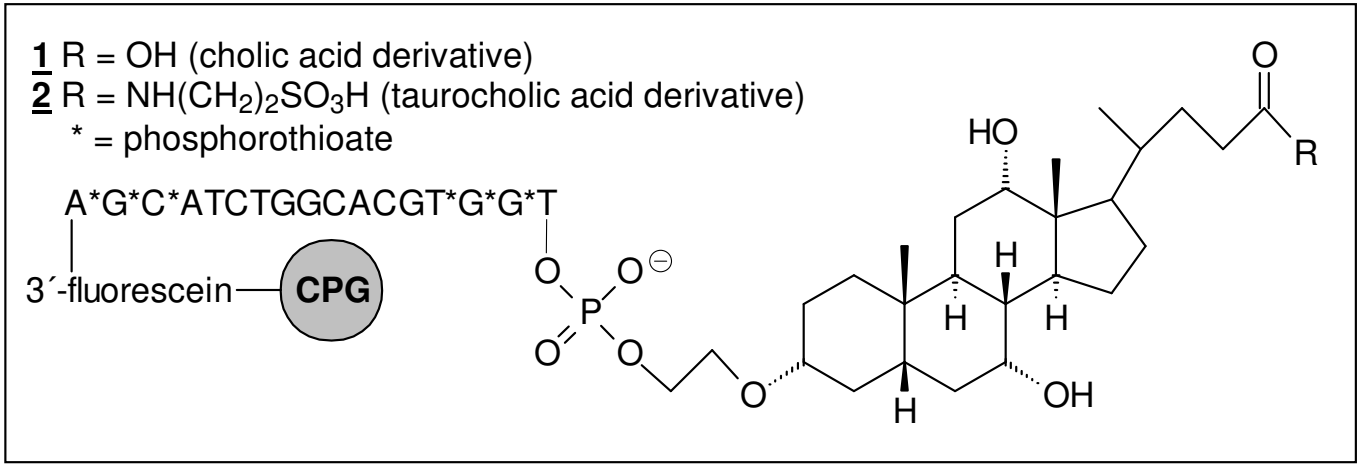

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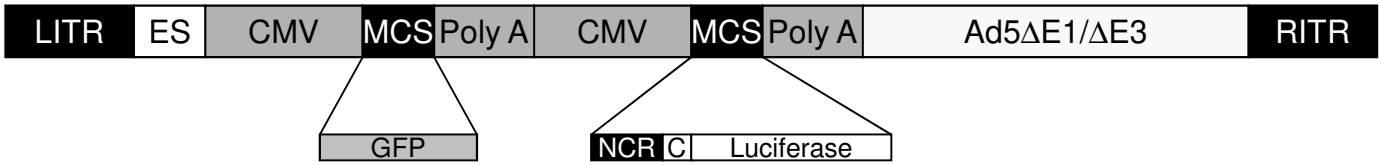

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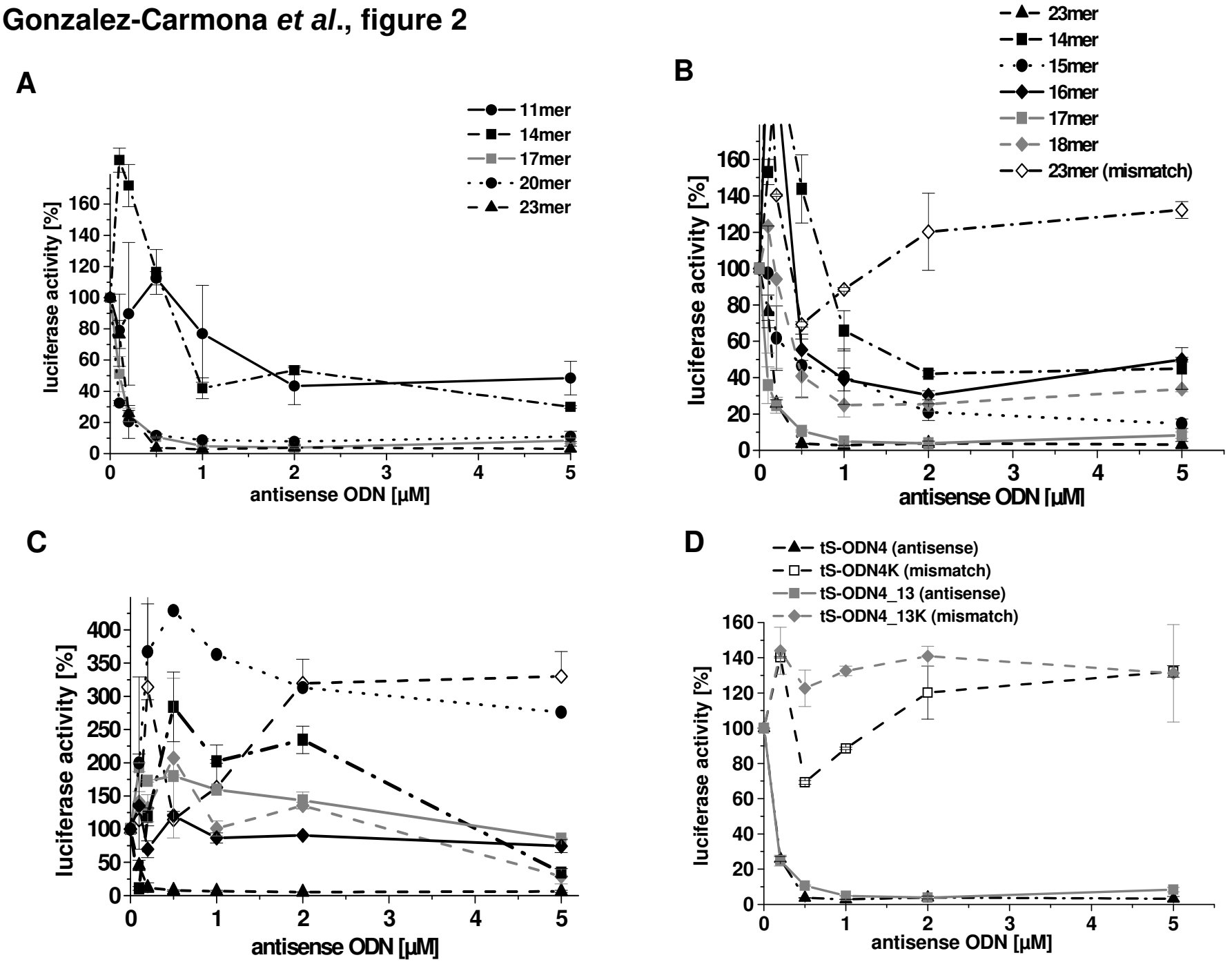

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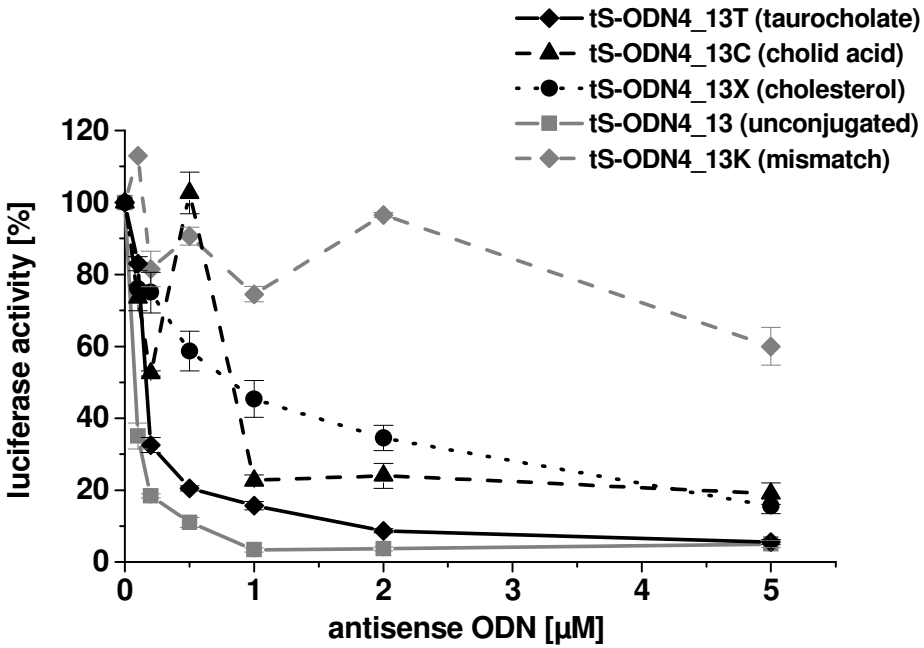

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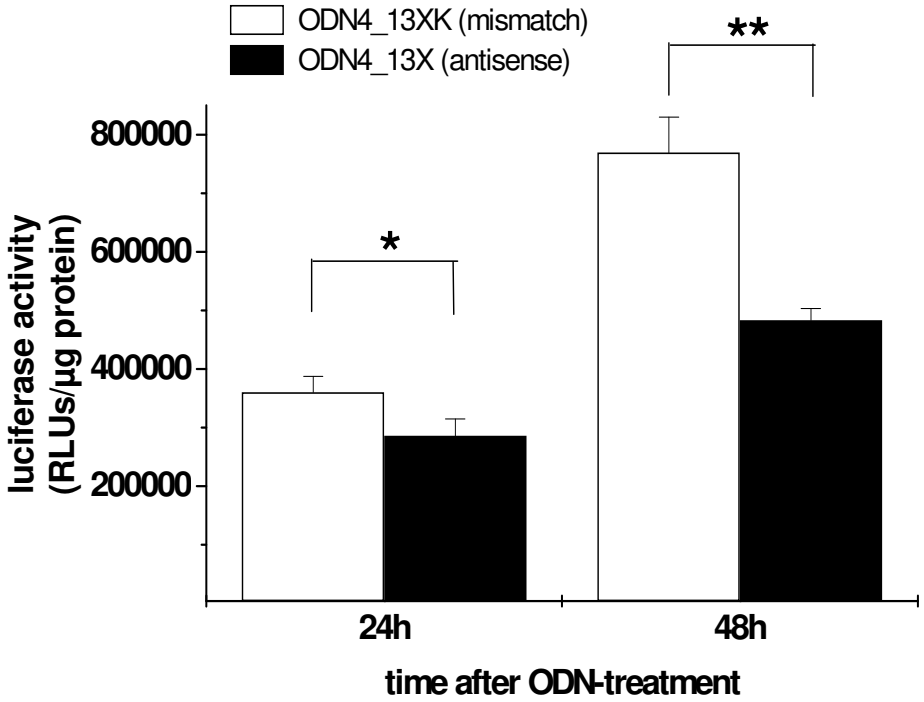

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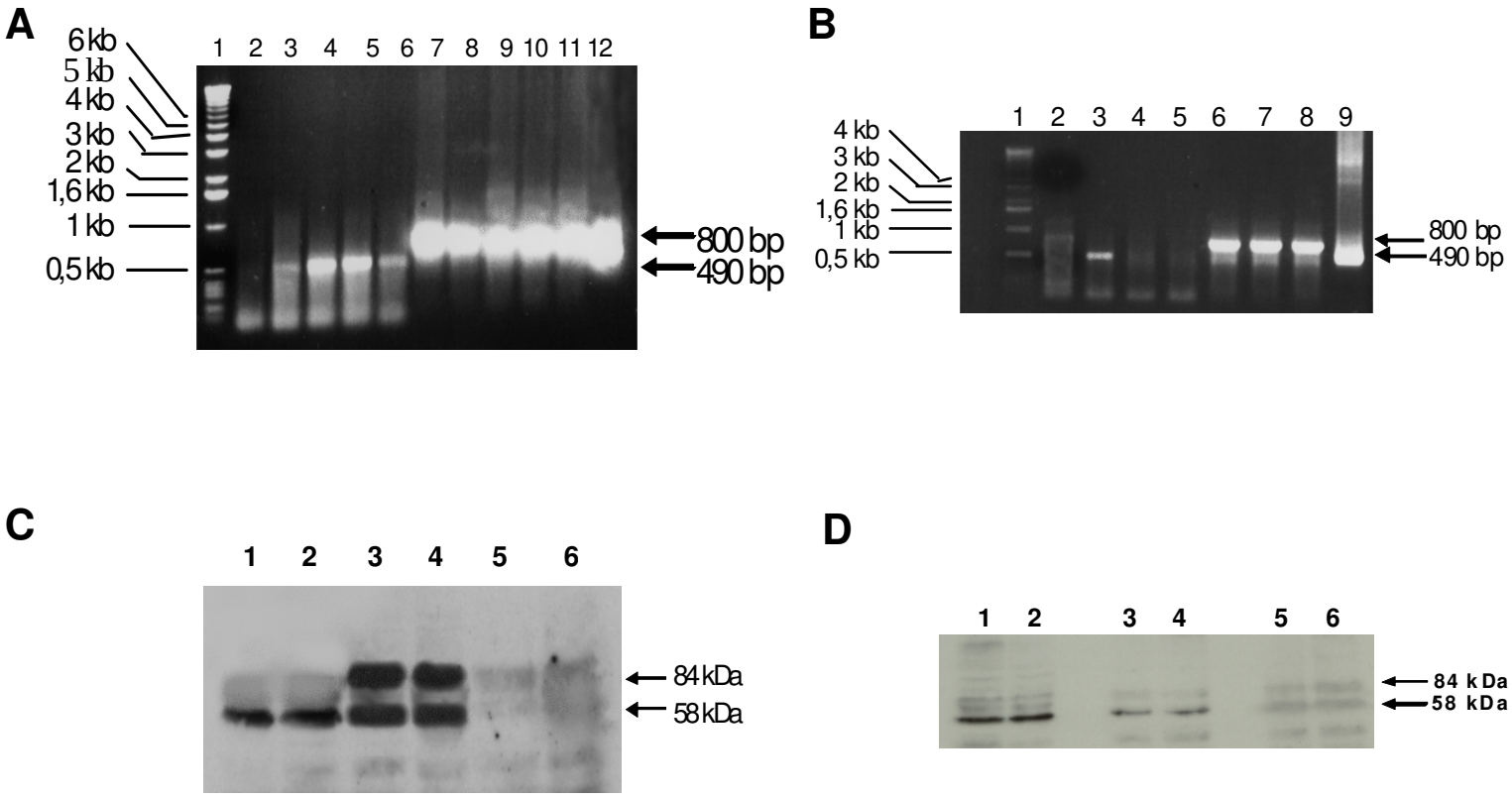

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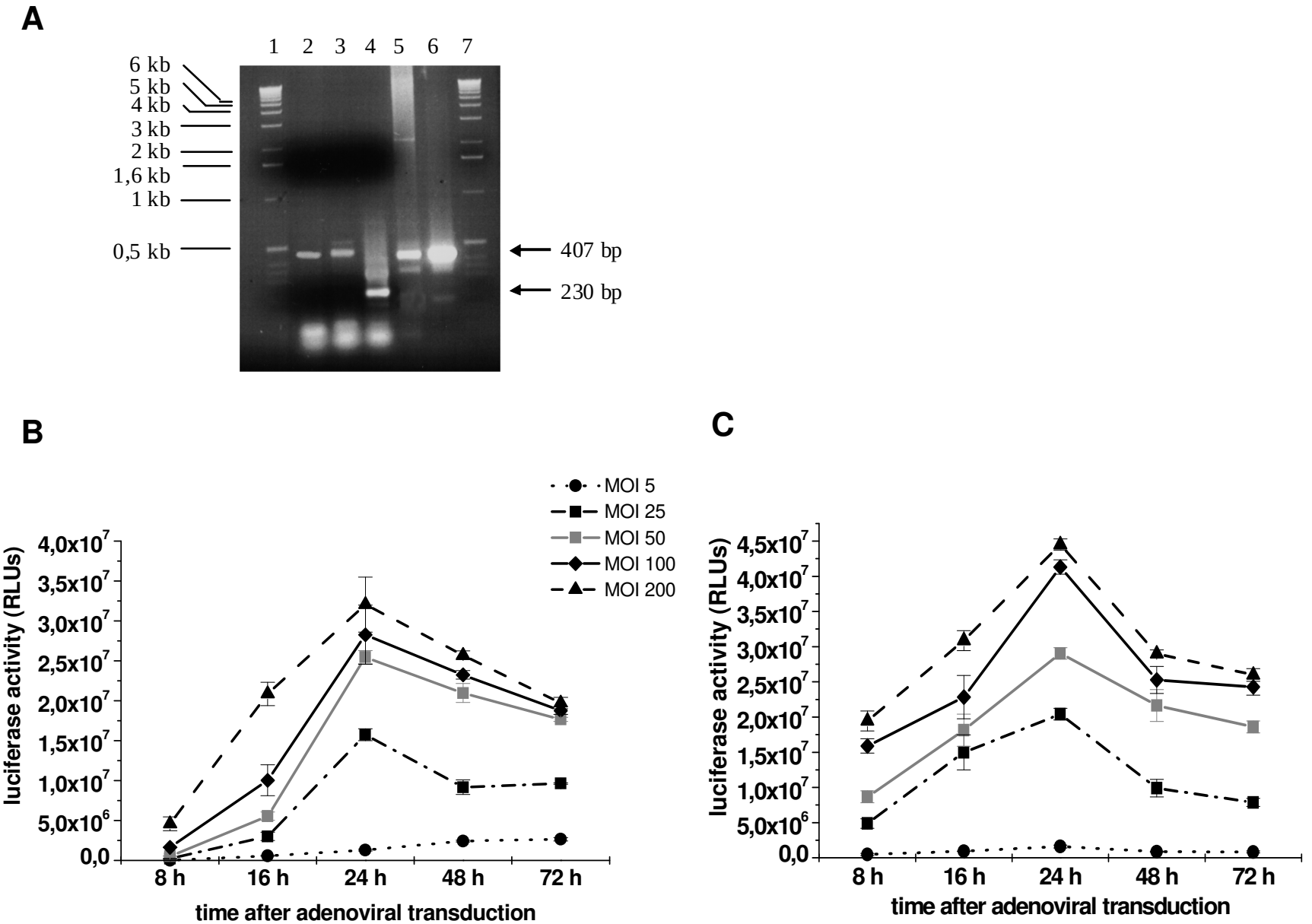

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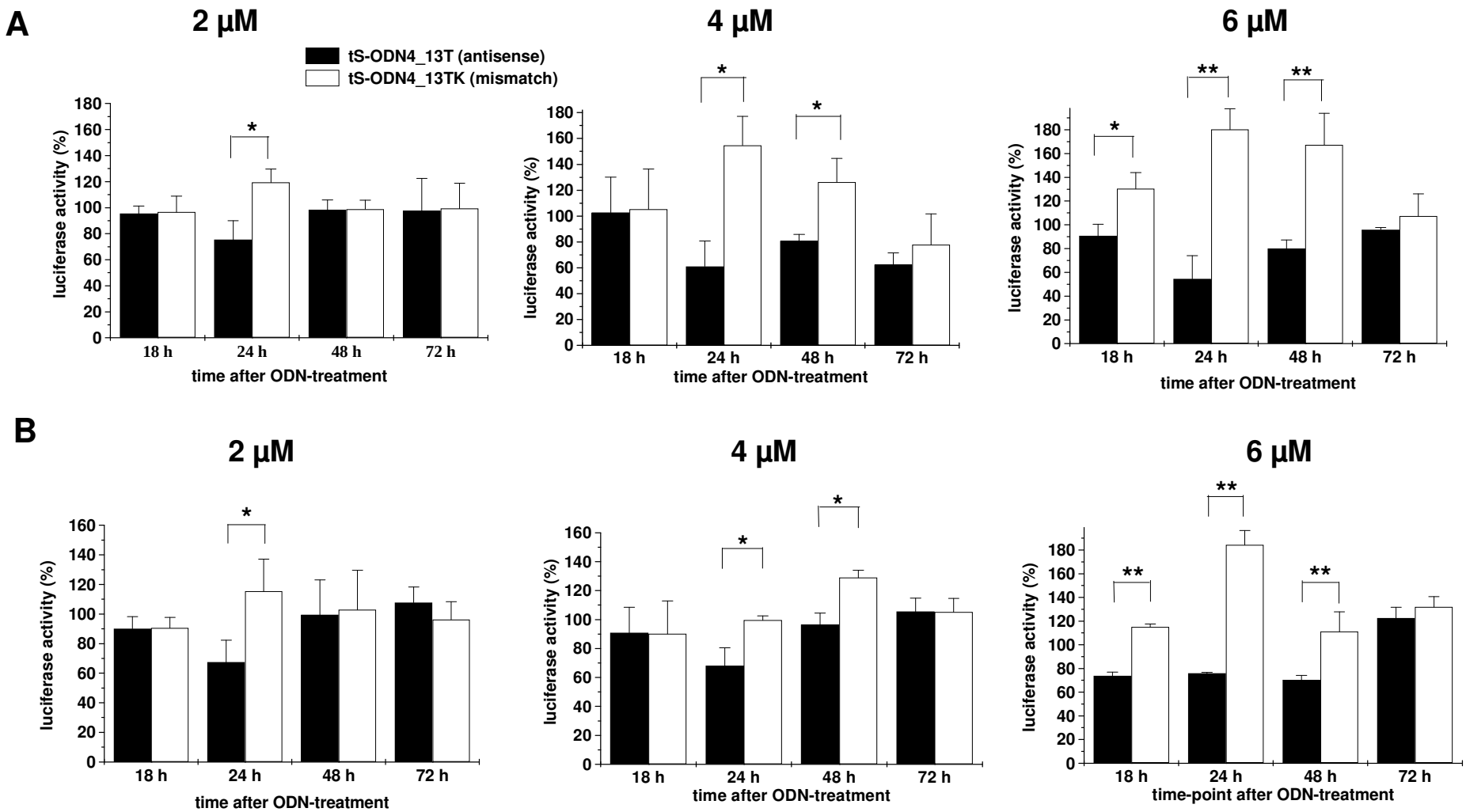

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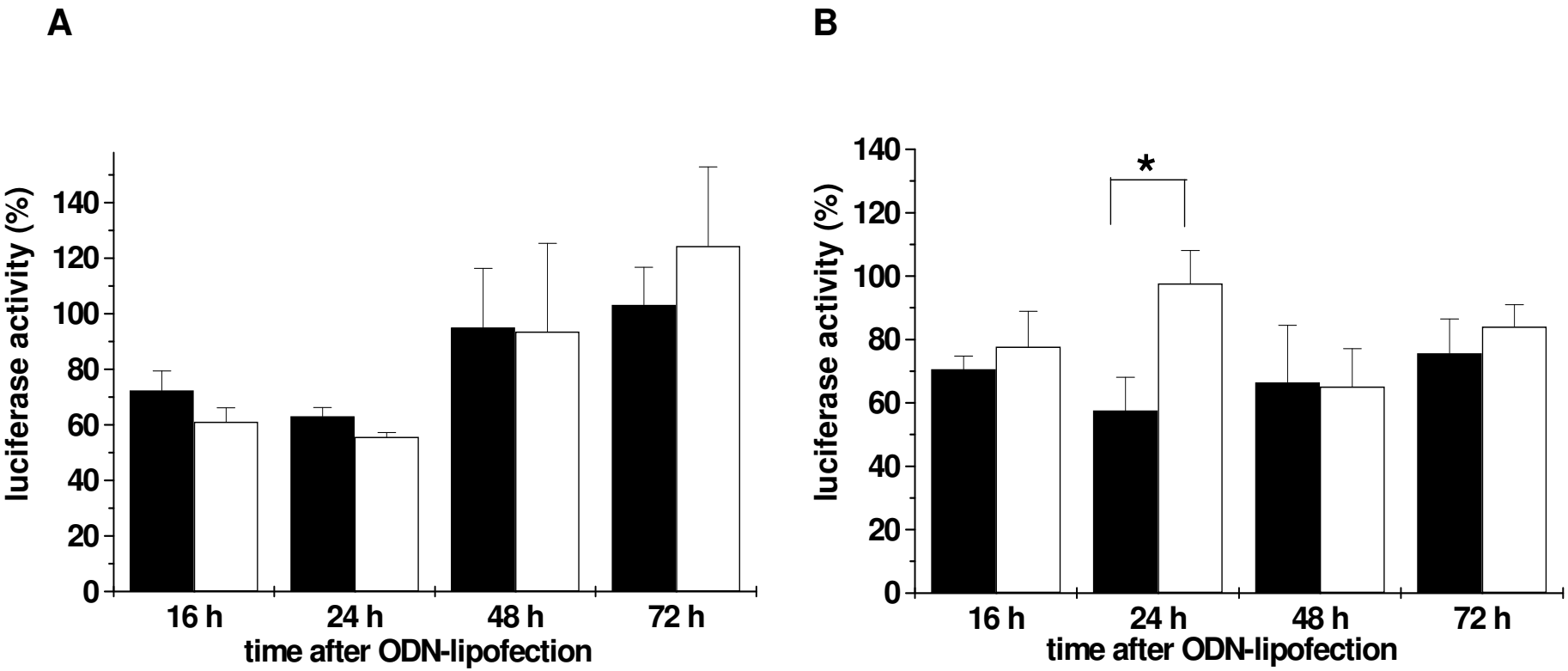
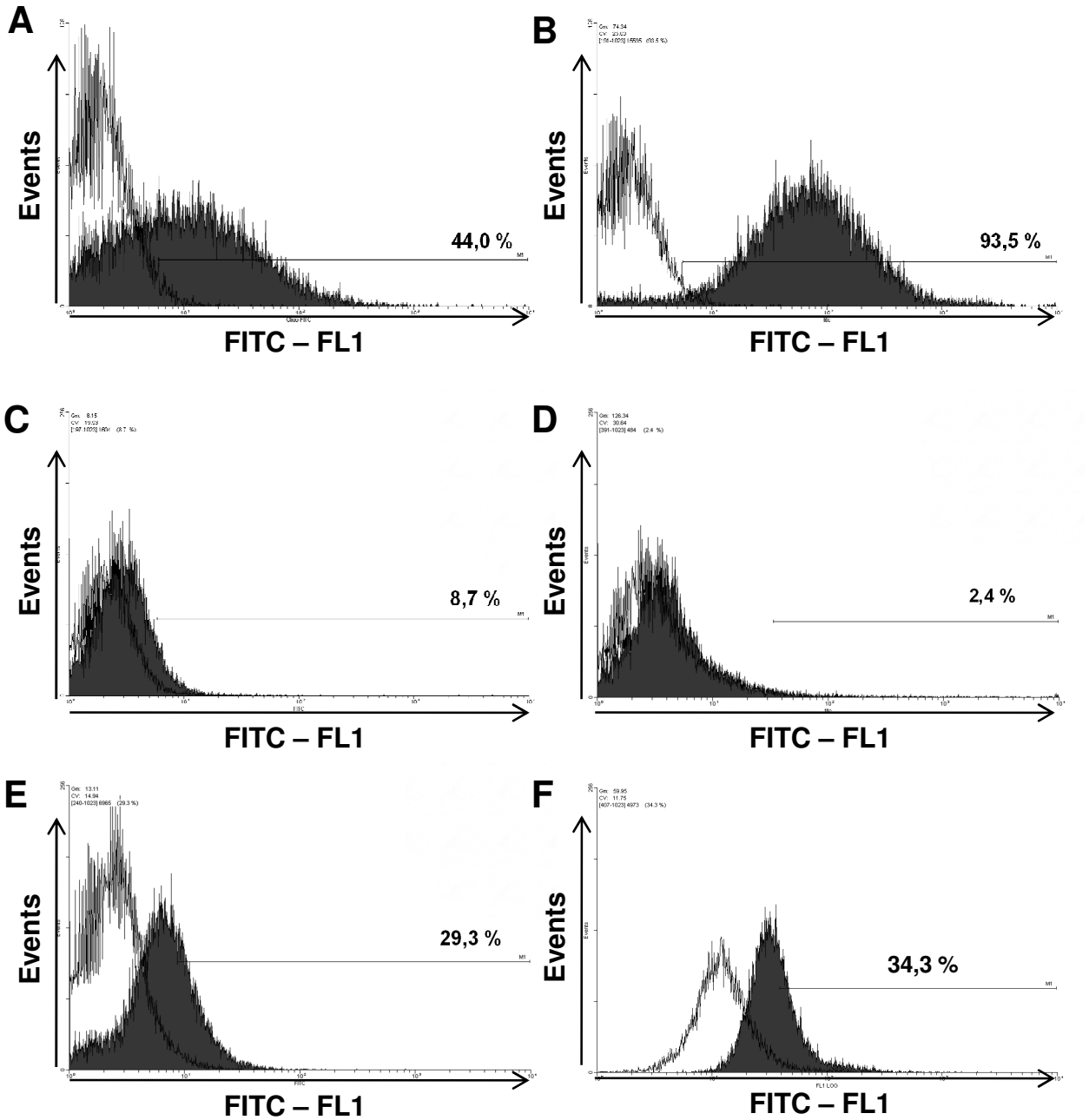
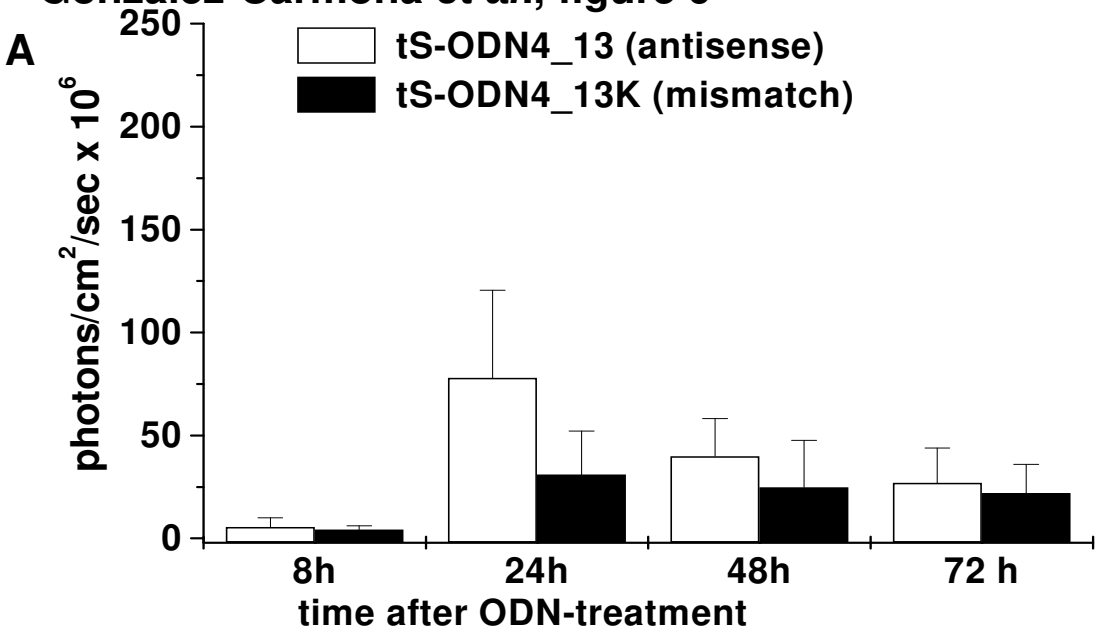
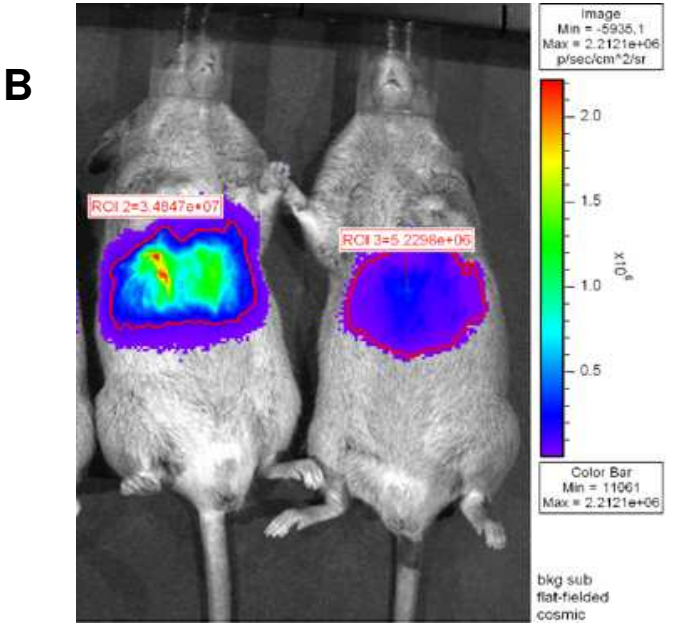


Gonzalez-Carmona *et al*., figure 8

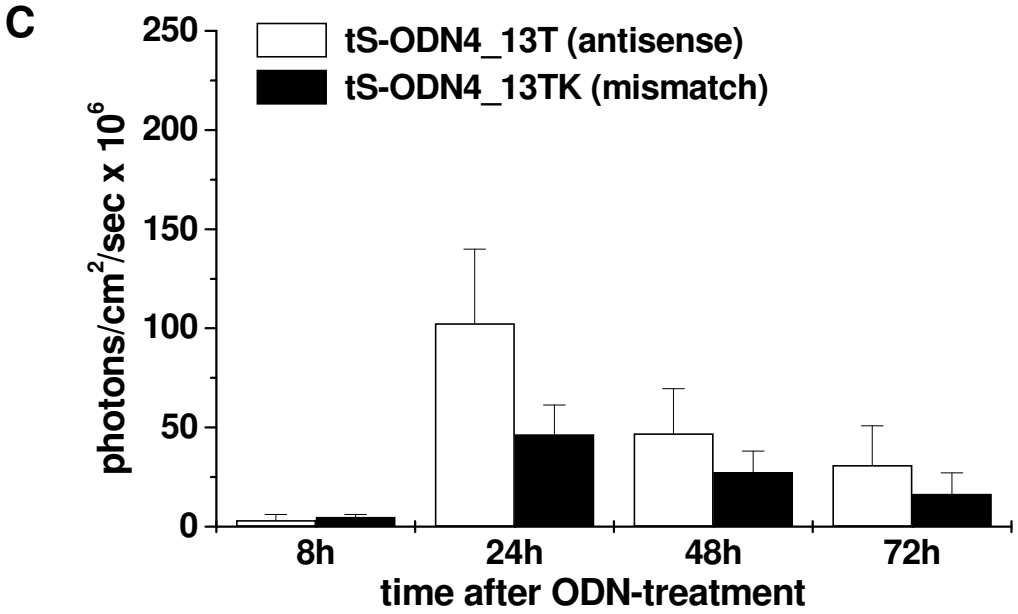


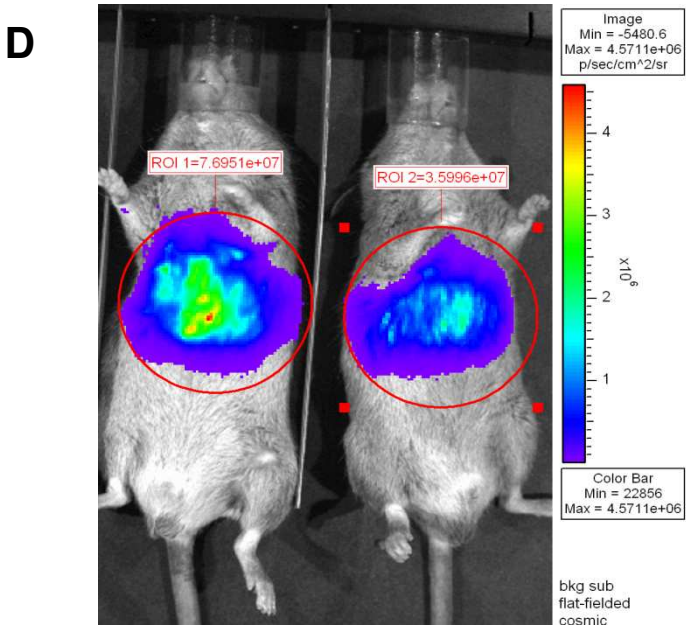
Gonzalez-Carmona *et al.*, figure 9





tS-ODN4_13K (mismatch) tS-ODN4_13 (antisense)





tS-ODN4_13TK (mismatch) tS-ODN4_13T (antisense)